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# microRNA-101 is a potent inhibitor of autophagy

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**Review timeline:**

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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 January 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, we experienced severe difficulties with the availability of suitable and willing referees during the past Christmas season.

Your manuscript has now finally been seen by three referees whose comments are shown below. As you will see while all three referees consider the study as interesting in principle and referee 1 is more positive overall it becomes clear that at this stage of analysis the study is not sufficiently developed to justify publication here. I will not repeat all their individual points of criticism, but the referees essentially all think that the functional link between miR-101 and the target genes stathmin and Rab5a and its functional significance needs to be analysed in considerably more depth. Furthermore, the effect of miR-101 on autophagy is not analysed in sufficient depth according to referees 2 and 3. Also, the choice of miRNA under study and the choice of miR-101 target genes is a matter of concern. Based on the referees assessment it thus becomes clear that the study is not publishable here at this stage of analysis.

Given the interest the study could spark in principle we would, however, be able to consider a new submission on the same topic should future studies allow you to strengthen the study considerably along the lines suggested by the reviewers and to develop the functional and mechanistic link between miR-101 and its targets further substantially. To be completely clear, however, I would like to stress that if you wish to send a new manuscript this will be treated as a new submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh (involving our original referees again if available at the time of resubmission), also with respect to the literature and

 

the novelty of your findings at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1

The manuscript by Lisa B. Frankel and collaborators, entitled "microRNA 101 is a potent inhibitor of autophagy" documents several main findings: 1) A number of microRNAs have an effect on autophagy regulation; 2) among them, miR-101 inhibits both basal and rapamycin-induced autophagy, mimicking beclin 1 downregulation; 3) two potential targets of this regulation are RAB5 and STMN1, recently involved in autophagy, and both downstream of mTORC1 regulation; 4) miR-101 tumor suppressor activity might be related, at least in part, to this regulation pathway.

This is an interesting paper, having the quality to bring to the attention of the autophagy research community three novel actors in the autophagy scenario: a miRNA with an hypothetic much larger target-set (as the authors, admittedly, state in the discussion) and two factors with oncogenetic potential.

The major flaw of this paper is the unclear choice of all these actors. A very superficial analysis of Figure 1B already tells the reader that the two other selected miRNAs (30c and 95) may have a stronger effect on autophagy, and another miRNA (unknown, or at least here undisclosed) has a much more dramatic effect on this process. When then checking Table 1, genes such POMP (proteasome maturation protein) and RCN2 (reticulocalbin) pops up as potential key factors on autophagy regulation (as degradation-involved or ER-regulated proteins, respectively), certainly more intriguing (and more dysregulated by miR-101) than RAB5 and STMN1. Last, a careful analysis of Supplementary Table 1 unveils ATG4D (as told by the authors in the Discussion paragraph; and with a fold change higher than that of APP, a demonstrated functional target of miR-101) and the major autophagy regulator ULK1 (with a fold change of 1,23). As regards STMN1, the authors base their choice mostly on unpublished data, ranging from alternative siRNA-based screens to the undisplayed observation that the levels of the protein change upon inhibition of lysosomal acidification. Although I am aware that large screening approaches give so many results that cannot be included in a single publication, the authors could put some efforts in better substantiating their selection, and indicate why some alternative miRNAs or target proteins were excluded by the analysis. The other way to see it, is that the present results are just by-products (even though quite relevant) of a much more interesting (and undisclosed) finding.

Another issue that is not clear regards the placement of miR-101 downstream of mTORC1. As visible in Supplementary Figure S2, miR-101 overexpression induces an increase of autophagy, which is higher upon etoposide treatment than after rapamycin addition (that is the most potent inhibitor of mTORC1). Can the author speculate about this discrepancy? Further, in Figure 5B, is evident a prominent effect of miR-101 overexpression in comparison with the interference of both STMN1 and RAB5, and even with that of the key pro-autophagic factor Beclin 1. This implies a much wider (or much more upstream) role for this miRNA in autophagy regulation.

Finally, I have a number of minor points:

1) In the introduction paragraph, the review articles cited when discussing about autophagy's role in development and diseases must definitely be updated to 2008-2010. Many exciting and all-round reviews have been published by opinion leaders in the last 2-3 years.

2) As a positive control in Figures 3A and 3B, Rubicon may have acted as a much more effective target than Raptor.

3) The efficiency of RAB5 downregulation by siRNA is low. Did the authors tried with different pairs of oligonucleotides?

4) There is an awkward use of punctuation all over the manuscript. E.g., see page 12, last paragraph, 4th row ("..., however interestingly...." )

Referee #2

In this interesting manuscript Frankel et al. describe regulation of autophagy by miR-101, the tumor suppressive miRNA, which was identified by a functional screening of microRNAs. Overexpression of miR-101 suppresses basal and induced autophagy, while reduction of endogenous miR-101 accelerates it. Thus, this microRNA is regarded as a negative regulator of autophagy. Next, the authors identified its targets and show that two of them, Stathmin and Rab5a inhibits autophagy. miR-101 directly targets them. Finally, they demonstrate that overexpression of miR-101 enhances cell death of MCF-7 induced by 4-OHT, which has been used in cancer therapy. Based on these results, the authors conclude that miR-101 negatively regulates autophagy via down regulation of Stathmin and rab5a protein level, and miR-101-mediated autophagy suppression sensitizes cancer cells to the anticancer drug-induced cell death.

The present manuscript is timely and of potential interest as it provides the first example of microRNA-mediated regulation of autophagy. However, the present report tempers the initial excitement, as the data provided in the manuscript do not fully support their conclusion. The most critical problem is lack of direct evidences showing that effect of miR-101 on autophagy is actually mediated by suppression of Stathmin and Rab5a. Moreover, the observed enhancement of 4-OHT-mediated cell death in miR-101-overexpressing cells lacks mechanistic explanation as well. There is no evidence that the effect is due to autophagy inhibition. The authors should show data linking the each phenomenon of autohagy inhibition, down regulation of Stathmin and Rab5a, and cell death enhancement. For example, do overexpression of the two target proteins suppress autophagy inhibition and cell death enhancement in miR-101-overexpressing cells?

In addition, to corroborate the conclusion, the authors should assess autophagy more rigorously. Besides puncta formation and degradation of LC3 and p62 accumulation, which have been done in the manuscript, quantification of LC3-II form in the presence or absence of protease inhibitors is required. The present data suggest that miR-101 inhibits autophagosome formation. Determination of which stage of autophagosome formation is regulated is very important. How about dot formation of Atg proteins upstream of LC3, such as ULK1 or Atg14 in miR-101-overexpressing cells? Wortmannin can reduce acceleration of autophagy by inhibition of endogenous miR-101? EM observation is also useful to see if abnormal autophagosome is formed. It is worthwhile and even obligatory to compare miR-101 inhibition and knockdown of Stathmin or rab5a about which stage of autophagy is inhibited, as the acting point should be same if miR-101 affects autophagy via control of these targets.

Referee #3

It is becoming clear that miRNAs have a big impact on shaping transcriptomes and proteomes of eukaryotic organisms; thus, their deregulation is closely linked to human diseases, such as cancer. Here the authors perform a luciferase-based functional screen in search for miRNAs that regulate

autophagy in cultured cell lines. They identify several miRNAs including miR-101 as potent inhibitors of autophagy. Among a number of candidate miR-101 targets, the authors study two genes, STMN1 and RAB5a, and show that these genes regulate autophagy. However, in their present form, they are far too preliminary for publication. For EMBO J to consider the work, I think the authors should need to gain further functional and mechanistic insight into the connection between miRNA-mediated regulation of gene expression and autophagy.

Major points:

miR-203 is used as a control. Where is miR-203 located in the plot of Figure 1B? Where in the plot of the Figure is the cutoff line drawn to identify candidate miRNAs that repress autophagy? The authors state in Figure S1 legend that "Highlighted in red are the wells transfected with the beclin-1 siRNA, generally with relatively high LC3WT/LC3G120A ratios." Do this comment and the results shown in Figure S1B indicate that this assay is not much reliable with low reproducibility?

Through this functional screening, the authors identify three miRNAs, miR-30c, miR-95 and miR-101, which inhibit the autophagic flux. The authors select miR-101 for further studies mainly because of its possible involvement in cancer development. As the authors state in Introduction and Discussion, miR-101 appears to have a large number of targets including EZH2 and genes listed in Table 1 and assayed in Figure 4C. It is already known that miR-101 overexpression, for instance, reduces cell proliferation (Varambally et al. 2008). Is the observed inhibition of autophagy by miR-101 overexpression due to the secondary effect of cell growth defect? Expression of miR-101 is often lost or down-regulated in several cancer types. Is autophagy enhanced in these cancer types? Does miR-101 overexpression affect the expression of miR-95 and/or miR-30c? It is known that miR-30a, which has the exactly same seed sequence as miR-30c, regulates beclin-1, a key autophagy-promoting gene (Zhu et al. 2009).

Other points:

1. Which step(s) of autophagy does miR-101 repress?

2. miR-101 overexpression reduces the expression of both Rab5a and Stemin1 (Figure 6D). Is double knockdown of rab5a and stemn1 additive with respect to autophagy inhibition (Figure 5)?

3. Among rab5 isoforms (a, b, and c), only rab5a has a miR-101 binding site in its 3'UTR. Is only rab5a isoform expressed in MCF7 cells? Is the antibody used for western blot specific for Rab5a (Figure 6D)? Does miR-101 overexpression affect the expression of other Rab5 isoforms?

Resubmission 08 July 2011

EMBOJ-2010-76692, Point-by-point response to reviewers:

<u>Improved screen analysis and main additional experimental work:</u>

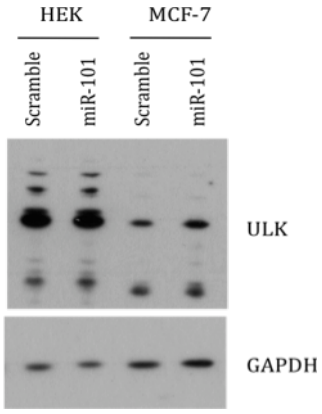
1. One of the main points of critique of the original version of this manuscript was that our choice of miRNA hits and targets for further analysis should be more clearly substantiated. We have now addressed these issues thoroughly:

    a. The previous depiction of the screen results (Fig 1B) showed only the result of 1 representative timepoint from 1 of 3 screens. To strengthen this figure, we have incorporated the results of all three screens into one graph (please see the revised version of Fig 1B). We have re-analyzed the data by ranking the miRNAs according to $LC3^{WT}/LC3^{G120A}$ values, allowing identification of statistically significant outliers common for all three screens by the product ranking method (see Materials and Methods). This analysis revealed a slightly different result, with the top hits being miR-95, miR-101 and miR-145. The latter two hits have, by far, the most well described links to cancer and since we have previously observed that miR-145 has undetectable expression levels in MCF-7 cells, we believe that this justifies our choice for continuing with miR-101. To be

completely clear – in Fig 1B, the individual dots on the graph that appear higher than miR-145, miR95 and miR-101, represent miRNAs that emerged as hits only in one of the three individual screens. Thus, we believe that they are technical outliers.

b. To address our choice of targets more thoroughly, we have now included a new Fig 5A where we have knocked down a larger panel of potentially interesting targets identified by the array (including several of those suggested by referee #1). Besides STMN1 and RAB5A, this led to the additional identification of ATG4D as an interesting target. Referee 1 also suggested testing ULK-1 as a miR-101 target. Although its mRNA is downregulated on the array, this gene does not contain a miR-101 binding site in its 3'UTR and is therefore unlikely to be a direct target. Accordingly, we do not observe a decrease in Ulk1 protein level upon miR-101 overexpression by western blotting (see figure below).




In general when analyzing miRNA effects by microarray, it should be kept in mind that changes on mRNA levels do not always reflect changes on protein levels (which are often stronger), and therefore the level of fold-change indicated on the array cannot be used directly to estimate how strong a target candidate is.

2. Importantly, we have now verified the functional significance of the identified miR-101 target, STMN1. As seen in Fig 8, overexpression of STMN1 partially rescues the miR-101 phenotype. Our focus on STMN1 was due to its novelty as an autophagy regulator, whereas roles for ATG4D and RAB5A in autophagy have been previously described in the literature. We must emphasize that we believe that miR-101 (like most other miRNAs), functions through a multitude of targets. Therefore, although STMN1 can account for some the effect, it is important to acknowledge (as also noted by the referee #1) that there are likely to be a number of other autophagy regulating targets including RAB5A and ATG4D, and possibly several others yet to be disclosed.

3. As suggested by the referees we have now included an electron microscopy analysis, which further confirms the miR-101 phenotype on autophagy. As can be seen in Fig 7 (and Fig S7) analysis of 25-30 cross-sectioned cells per sample showed a clear reduction in the number of autophagosomes upon miR-101 overexpression. Inhibition of autophagy by miR-101 has now been confirmed by 4 independent assays in this manuscript, and we therefore believe that the evidence for this phenotype is very strong. Addtionally, EM was used to confirm the phenotype for siSTMN1.

<u>Other additional data</u>

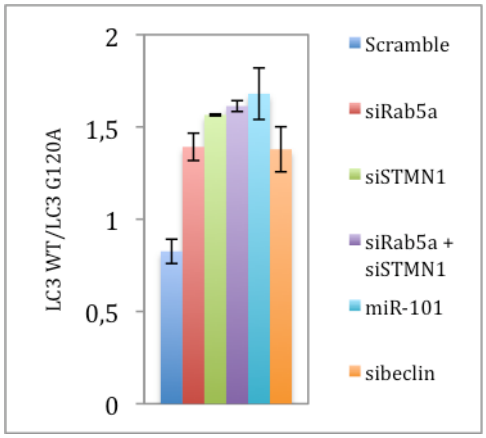
1. Fig 6 now includes additional data showing that ATG4D, like STMN1 and RAB5A, also is a direct miR-101 target.

2. To address the concern from referee #3 about whether the effect of miR-101 on autophagy could be secondary to a growth defect, we have included Suppl Fig S4. This figure

demonstrates that depletion of miR-101 has, despite its pronounced effect on autophagy, no observed effect on cell growth.

3. Fig 5B has been updated. The experiment was repeated in order to include the effect of ATG4D knockdown on autophagic flux. In this experiment, miR-101 has a stronger effect than the individual siRNAs (even more so in the previous version of this figure) and as pointed out by referee #1, this could indicate that miR-101 has broader effect on autophagy. Also, with regards to this issue, and as suggested by referee #3, we have tested the combined effect of siSTMN1 and siRAB5A on autophagic flux. As can be seen from the RLuc flux assay below, there does not seem to be an additive effect of these siRNAs, suggesting that these two proteins function within the same signaling pathway




4. In support of our indications for a function for miR-101 downstream of mTORC1, we have now included Suppl Fig S6, showing that miR-101 can override the effect of the siRNA against Raptor.

Smaller points, comments and clarifications:

Referee 1:

1. We would like to point out that suppl fig. S2 was previously misinterpreted by referee #1. This figure shows that levels of endogenous miR101 (measured by miRNA-specific qPCR) increase upon induction of autophagy by various autophagy inducers. Therefore, this figure has nothing to do with our placement of miR-101 function downstream of mTOR, but merely suggests a physiological role for endogenous miR-101 in autophagy.

2. We have updated the review articles cited in the introduction.

3. We do not see the need for a better positive control than siRNA against raptor as the effect (Fig 3A,3B,S6) is in our opinion quite clear. Also the knockdown efficiency of this siRNA is very good (Suppl Fig S5A)

4. Regarding Rab5a siRNA efficiency, we tested two other siRNA sequences. However, we were not able to obtain a better knock-down that that shown in Suppl Fig S5B.

Referee 2:

We agree with the referee that our 4-OHT experiments (Fig 9) do not unequivocally prove that miR-101 collaborates with 4-OHT in inducing cell death by targeting autophagy. We have therefore rephrased our conclusions concerning this figure. We believe we have made a very strong case for miR-101 as an autophagy inhibitor and a large bulk of literature demonstrates collaborativity in cell killing when combining autophagy-inducing chemotherapeutics with inhibitors of autophagy. In addition, we do not see how this concern could be experimentally addressed in a satisfactory

manner, as it would require that we could either re-activate autophagy downstream from miR-101 or rule out a function in cell death of all miR-101 targets not regulating autophagy.

Referee 3:

1. miR-203 was chosen as a control because this miRNA does not have any effect on autophagy. This is clear from Fig 2A, 2C and 5A, where miR-203 does not deviate from other negative controls such as the Scramble and Renilla siRNAs, underlining why this miRNA control was suitable.

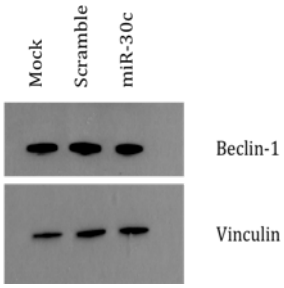
2. Concerning cut-off and definition of outliers, please see the updated screen analysis and underlying statistics described in materials and methods.

3. Regarding Suppl Fig S1B: It is true that there is an apparent variation in the efficiency of the beclin-1 siRNA at different positions on different plates (note however that this figure shows only one timepoint from one screen and therefore does not tell us anything about reproducibility between screens). Plate by plate variation as well as variation at different positions within the same plate are often an issue in large-scale screens, stressing the need for biological replicates, stringent statistics and a thorough validation process, all of which have been of very high priority in our screen and follow-up analysis. Figure S1B can alternatively be removed for clarity.

4. The question of whether autophagy is enhanced in cancer types where miR-101 is lost is very interesting, however also very tricky to address due to the many well-known technical difficulties in quantifying autophagy *in vivo*.

5. Interregulation between miRNAs is an interesting concept. However, since the effects of miR-101 are seen both upon its knockdown and overexpression, our results are not likely to be secondary effects of other miRNAs.

6. It is true that miR-30a (same seed as miR-30c which was a hit in the original screen analysis) is published to regulate Beclin-1. Naturally, we tested whether miR-30c also could regulate Beclin-1. However, we did not see a convincing reduction in Beclin-1 protein levels by western blotting when overexpressing miR-30c (see below).




7. The antibody used for Rab5 recognizes all three Rab5 isoforms, however based on our array data MCF-7 cells express significantly higher levels of 5a relative to 5b and 5c isoforms (see below):

Average (log2(expression)) in scramble treated samples from the array:
RAB5A: 11.83894
RAB5B: 9.266181
RAB5C: 10.00957

This means that RAB5A is 5.95 times higher than RAB5B, and 3.55 times higher than RAB5C
--> rab5a/rab5b = 2^(11.83894-9.266181) = 5.95
--> rab5a/rab5c = 2^(11.83894- 10.00957) = 3.55

More importantly, miR-101 does not affect the expression of the 5b or 5c isoforms significantly (see array data below), fitting well with the fact that these two isoforms do not contain seed matches in their 3'UTRs:

RAB5A fold change: 1.91, adj. Pval: 3.119959e-10
RAB5C fold change: 1.17, adj. Pval: 1.935381e-01
RAB5B fold change: 1.08, adj. Pval: 5.312863e-01

A final point of importance was mentioned by referees #2 and #3 who asked for more specificity regarding at which stage of autophagy miR-101 and its targets function. We show that overexpression of miR-101, as well as depletion of its targets STMN1, RAB5A and ATG4D inhibit both LC3 translocation and autophagic flux, and therefore it seems that the block is located upstream LC3 lipidation (Fig 5A,5B). This block is also apparent for Rapamycin-induced autophagy, strongly suggesting that miR-101 functions downstream of mTORC1 (Fig 5A). Additionally supporting this, miR-101 effectively inhibits siRaptor-induced autophagy (Fig S6). Therefore we assume that the main functions of miR-101 targets are downstream mTORC1 and upstream LC3 lipidation. This fits nicely with published data for RAB5A and ATG4D, however other miR-101 targets including STMN1 could theoretically have several different modes of action. In deed, Fig 8B may suggest a function for Stathmin downstream Beclin-1, since the Beclin-1 siRNA is still effective in Stathmin overexpressing cells (no significant difference rel. to control cells).

2nd Editorial Decision 01 August 2011

Thank you for sending us a new version of your manuscript, previously EMBOJ-2010-76692. Our original referees 1 (now referee 1) and 3 (now referee 2) have now seen it again. In general, the referees are now positive about publication of your paper. Still, both referees think that that there are a few remaining minor issues that need to be addressed (see below). I would therefore like to ask you to deal with the issues raised in an amended version of the manuscript. With respect to point 2 of referee 2 let me point out that at The EMBO Journal we encourage the citation of primary literature rather than review articles wherever possible.

In addition, there are a number of editorial issues that need further attention:

Please add an author contributions section as well as a conflict of interest statement to the main body of the text, after the acknowledgements section.

Please include the details for the public database entry of your microarray data into the main body of the text.

Please add scale bars and explanations to all figures containing microscopic pictures including figure 2B, 3B, 9B and 9E.

Please add the statistical details to the legend of figure S4.

Please let us have a suitably amended manuscript as soon as possible. I will then formally accept it.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1

In this manuscript, Frankel et al explore molecular pathways leading to autophagy. The authors show that a tumor suppressive microRNA, miR-101 acts as a potent inhibitor of autophagy. They also demonstrate that miR-101 is induced by various triggers of autophagy (starvation, rapamycin, and etoposide treatment) and regulates the expression of STMN1 (Stathmin), RAB5A, and ATG4D. The authors further demonstrate that miR-101, likely through its inhibition of autophagy, can sensitize breast cancer cells to the active metabolite of tamoxifen, 4-hydroxytamoxifen (4-OHT)-mediated cell death. This manuscript contains some interesting results. However, the paper will be considerably strengthened by additional data and by addressing the points detailed below.

1) Can depletion of miR-101 target genes such as Stathmin also synergize with 4-OHT in MCF-7 cell?

Trivia:

1) In legend of Supplementary Figure S2: qpcr should be qPCR.

Referee #2

The authors have significantly improved the paper, in particular as regards the real relevance of miR-101, in comparison with other screened autophagy-related miRNAs. I have no more concerns about this, that was the major flaw of the manuscript.

In general, the paper is an outstanding report which highlights the first strong involvement of a miRNA in autophagy regulation.

Thus, I believe it will be highly cited in the next future.

As minor 'suggestions':

1) As regards the link between supplementary Figure S2 and the conclusion that mTOR is upstream of this regulation, I may well have misinterpreted it. The issue, however, is still whether they can give some explanations about their finding (Figure S2B, top) that rapamycin (mTOR regulator) works on the miRNA regulation at a lesser extent than etoposide. This, on my opinion, still tells us that also other signals (maybe different from mTOR) have the potential to upregulate this key miRNA. They could add this option as a small sentence where they like it more.
2) Last, the introduction still presents review articles from 2003 or 2004 regarding developmental autophagy. In one case (Kuma et al.), they refer to a paper discussin' the perinatal importance of Atg genes; in the other they cite (Yue et al., 2003) a single paper on Beclin 1 knockout. Either the authors cite specific works (that may well be dated to those years), or they update the review literature, which is, I believe, much more useful to the reader besides adding data on other relevant defects.

1st Revision - authors' response 16 August 2011

Please receive a final revised version of our manuscript (EMBOJ-2011-78719). Since our previous submission we have addressed the issues raised by you and the referees as follows:

- An author contributions section and a conflict of interest section have been added.

- The details for the public database entry of our microarray data have been added.
- Scale bars have been added to figures 2B, 3B, 9B and 9E.
- Statistical details have been added to figure legend S4.

Regarding comments from referee #1:

1. The question of whether depletion of miR-101 target genes can synergize with 4-OHT would be interesting to address, however we do not believe that this experiment would add significant value to the quality of our manuscript. Also, the time needed to perform these experiments would cause a significant and unwanted delay in the publication process.
2. qpcr has been changed to qPCR in supplementary figure legend S2

Regarding comments from referee #2:

1. A sentence regarding differential effects of etoposide and rapamycin on miR-101 expression has been added to the results section.
2. The two mentioned references Yue et al. and Kuma et al. refer to important original articles reporting the first Beclin-1 and ATG5 knockout animals. These papers were published in 2003 and 2004, respectively, and we find it more appropriate to cite the primary literature rather than newer review articles.

Please note that the total character count for this manuscript is now 55,893 characters including title page, abstract and figure legends, but excluding references, tables and supplementary material. We hope that this will be acceptable and we look forward to hearing from you.

 